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14. ABSTRACT
This project evaluated the brain tourniquet concept, a new therapeutic approach to battlefield traumatic brain injury (TBI) that aims to enhance warfighter protection by fulfilling two main goals: 1) protect uninjured brain regions against the spread of secondary damage, such as excitotoxic cell death, and 2) maintain normal function in the remaining intact brain circuitry, possibly by physiologically isolating damaged brain regions. Experiments employed a rat brain slice model of superficial neocortical TBI. Randomly selected damaged brain slices were treated after injury with either a nootropic agent (aniracetam, cyclothiazide, IDRA 21, or 1-BCP) or the antiepileptic drug levetiracetam. Our results showed that IDRA 21 protected damaged slices against trauma-induced cell death and preserved normal neural function in the remaining intact neocortical regions. IDRA 21 inhibited post-traumatic epileptic activity by significantly raising seizure threshold and preserved the ability of damaged cortices to support long-term potentiation of synaptic transmission. Histological analysis showed IDRA 21 prevented injury-induced cell death, significantly reducing levels of necrotic neurons. Levetiracetam was superior to IDRA 21 in inhibiting trauma-induced epileptogenesis, but less effective in protecting against cell necrosis. These results support the feasibility of the brain tourniquet concept and its potential development via a pharmacological approach.

15. SUBJECT TERMS
traumatic brain injury, cell necrosis, neuroprotection, nootropics, epilepsy, long-term potentiation, synaptic transmission, electrophysiology, Fluoro-Jade B, histochemistry

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Introduction

This report summarizes the work performed between September 1, 2007 and February 29, 2008 for the Air Force Office of Scientific Research and the DARPA Defense Sciences Office. The contributors to this effort were Dr. Douglas S. F. Ling (Principal Investigator; Assistant Professor, SUNY Downstate Medical Center), Dr. Lie Yang (investigator; Research Assistant Professor, SUNY Downstate), Chang-Chi Hsieh MS (graduate student, Neural and Behavioral Science Program, SUNY Downstate), and Sonia Afroz (research assistant; undergraduate student, Brooklyn College).

The objective of this study was to assess the feasibility of the "brain tourniquet" concept, a new therapeutic approach to battlefield traumatic brain injury (TBI). The purpose of the brain tourniquet is to physiologically isolate damaged brain regions in order to allow head-injured warfighters to remain combat effective after sustaining brain trauma. This will also enhance recovery from this type of injury by preventing the spread of damage into otherwise healthy brain tissue and extending the "golden hour" to reach comprehensive medical care. As such, the ultimate goal of the brain tourniquet is two-fold: 1) physiologically isolate injured brain regions to halt (or at least delay) the spread of neurological damage to uninjured brain areas, such as excitotoxicity, and 2) maintain or enhance normal physiological function in the intact, uninjured brain regions to preserve any remaining cognitive and motor function. This dual requirement is dictated by the unique demands of the battlefield environment, in which incapacitation of brain function renders the warfighter completely defenseless. The critical feature of this approach is that, if successful, it will enable head-injured soldiers to continue to function in the combat setting (i.e., for self-defense) until comprehensive medical care or medical evacuation can be effected.

In this study, we evaluated the effectiveness of a pharmacological approach based on timely, post-injury interventions with putatively neuroprotective drugs. This approach was based on past studies by our laboratory which showed that rapid, post-injury treatment of injured rat brain slice preparations with anticonvulsants can prevent the development of neuropathologies, such as epileptic activity [Yang and Benardo, 2000]. However, many anticonvulsant agents produce profound, disabling cognitive impairments that would render their use problematic in an effective brain tourniquet system. We chose to focus our investigations on the nootropic (cognition enhancing) drugs, which are positive allosteric modulators of glutamateric α-amino-3-hydroxy-5- methyl-4-isoxazole- propionate receptor (AMPAR) function. The nootropics have been shown to enhance cognition and nemonic function in a variety learning paradigms [Davis et al., 1997; Granger et al., 1996; Granger et al., 1993; Shors et al., 1995]. In addition, reports indicate nootropics may be neuroprotective against excitotoxic and ischemic brain injury [Pizzi et al., 1995; Roger et al., 2004]. We have found that nootropics also enhance GABA-mediated inhibitory events, which would serve to dampen excess excitation and inhibit excitotoxic activity [Ling and Benardo, 2005]. As such, the nootropics may hold promise to meeting the dual objectives of the brain tourniquet approach.

Four well-known nootropic agents were evaluated: **aniracetam**, a pyrrolidione analog that slows non-NMDA (AMPA/kainate) receptor desensitization and deactivation [Isaacson and Nicoll, 1991; Ito et al., 1990; Tang et al., 1991], and may also directly enhance GABAegic transmission [Ling and Benardo, 2005]; **cyclothiazide**, a benzothiazide that is a potent inhibitor of AMPAR desensitization [Pizzi et al., 1995; Yamada and Tang, 1993] but has little effect on receptor deactivation [Arai and Lynch, 1998]; **IDRA 21**, a benzothiazide that is superior to cyclothiazide in vivo in crossing the blood-brain barrier, enhancing synaptic transmission, and promoting cognition [Arai et al., 1996]; **1-BCP**, a benzoylpiperidine that has been shown to improve cognition in rats [Stäubli et al., 1994], and has more potent effects than aniracetam in rat brain slices [Arai et al., 1994].

In supplemental experiments that were added to the study subsequent to the original proposal, we examined **levetiracetam**, a structural analog of the pyrrolidione ("acetam") nootropics that is also a potent anticonvulsant used clinically as an FDA-approved treatment for seizures and epilepsy [Lynch et al., 2004]. Given its relationship to the nootropics and its proven clinical efficacy in controlling seizures, levetiracetam appeared to be another good candidate for the brain tourniquet system.

Each agent was each assessed on its ability to isolate injured brain regions by preventing the development of neuropathophysiologies, such as seizure-like discharges, and the spread of necrotic cell damage. The hypothesis was they would do so without impairing normal neural function in the remaining intact regions, i.e., in effect creating a brain tourniquet. If successful, the brain tourniquet principle could be extended to remedy other conditions that would benefit from the selective isolation or shut-down of specifically targeted brain regions, such as schizophrenia, epilepsy, or even sleep-deprivation.

Specific Aims

Electrophysiology and histology experiments were used to assess cortical physiology and cell survival, respectively, in acute coronal slice preparations of rat neocortex that were maintained in vitro. The experiments were designed and conducted in the context of three specific aims, each of which directed at evaluating drug efficacy on three separate levels of neural protection, as dictated by the dual requirements of the brain tourniquet approach. Specific Aims 1 and 2 addressed the potential of candidate drugs to halt the spread of pathological sequelae of brain injury. Specific Aim 3 addressed the ability of drug interventions to preserve the physiological processes that support normal cognitive operations. These main aims are presented as follows:

1. Does post-injury, nootropic drug treatment of damaged brain slice preparations prevent or inhibit abnormal physiological activity, such as epileptic discharges?

2. Does post-injury, nootropic drug treatment of damaged brain slice preparations prevent or reduce necrotic cell loss?

3. Does post-injury, nootropic drug treatment of damaged brain slice preparations preserve the normal physiological processes that support cognitive operations, such as activity-dependent long-term potentiation (LTP)?

The first level of testing evaluated the candidate agents for effectiveness in preventing or inhibiting trauma-induced neuropathophysiologies, specifically epileptiform (seizure-like) activity. This was assessed through the use of electrophsyiological recordings in neocortical brain slice preparations. Since a key requirement of the brain tourniquet approach is to preserve normal neurophysiological function, this first test served as the primary screen for drug efficacy.

The second level of testing examined the ability of candidate drugs to prevent or inhibit the spread of trauma-induced necrotic cell damage to intact cortical areas, thus isolating neural damage to the immediately traumatized regions. Histological analysis of cortical slices was performed with Fluoro-Jade B (FJB), a fluorescent marker that specifically labels necrotic neurons [Schmued and Hopkins, 2000]. Cell counts of FJB-positive cells in slices were used to assess the levels of cell necrosis in drug-treated and non-treated brain slice preparations.

The third level of tests assessed the ability of candidate drugs to preserve the normal physiological processes that support normal cognitive operations, such as memory. Electrophysiological recordings were used to assess whether damaged brain slices treated with each drug could support activity-dependent long-term potentiation (LTP) of synaptic transmission, the form of synaptic plasticity widely believed to be the physiological substrate of learning and memory.

Experimental Design: In Vitro Studies of Traumatic Brain Injury

We used an *in vitro* rodent brain slice preparation that models severe, penetrating brain injury to the neocortex, such as those caused by blast-generated shrapnel and missile wounds. Epidemiological studies of TBI have shown that superficial cortical damage is a feature shared by several independent risk factors of brain injury including penetrating wounds, subdural hematoma, epidural hematoma, and depressed skull fracture, all of which are known precipitants for seizures and other neuropathologies [Annegers et al., 1998]. In addition, superficial cortical damage occurs with extra axial lesions that result from other diseases that carry high rates of brain pathology, such as meningiomas. The model used in this study was originally developed in our laboratory [Yang and Benardo, 1997] and simulates damage to the superficial cortical layers. It utilizes coronal slices of rat somatosensory cortex that are experimentally injured by removal of the superficial third of the cortex (i.e., layers I, II, and part of III). The model has several features which make it ideal to study the effects of neurotrauma on cortical physiology: (a) it simulates damage inflicted by a superficial penetrating or shearing head wound, (b) it is highly reproducible in yielding pathophysiologies in 55-60% of preparations, such as epileptic activity, and (c) once established, these pathophysiologies persist for the duration of the experiment (i.e., several hours). Although this preparation is a reductionist model that lacks many important features of CNS trauma in vivo, it serves as a useful complement to in vivo models by allowing detailed examination of injury-induced changes in cellular and network processes without the complications of systemic effects. It is a prime simulation of any lesion targeting the superficial cortex.

Three separate neocortical slice preparations were used: 1) "intact"slices that were not traumatized; 2) "damaged" slices that were traumatically injured by removal of superficial third of neocortex, but received no drug treatment; 3) "treated damaged"slices that were traumatized and then treated with one of the candidate neuroprotective drugs. All drugs were administered to slices within 30 min after injury via the bathing medium and continuously applied for 1 h, after which they were washed out with standard physiological saline.

We had originally proposed to use whole-cell, patch-clamp techniques to record from neocortical neurons, but found that these techniques were not optimal for this study, because persistent, injury-induced epileptogensis could not be reliably observed in neurons under whole-cell access. This was may have been due to whole-cell dialysis of target cells washing out cytoplasmic factors essential for maintaining plastic changes in neurons [Malinow and Tsien, 1990; Ling et al., 2002]. Consequently, electrophysiological experiments were performed using standard "sharp-electrode" intracellular techniques and extracellular field recordings. Upon completion of electrophysiology experiments, slices were recovered, fixed, and processed for histological analysis.

A detailed description of the specific experimental methods used in this study is provided in the following section.

Experimental Methods

Preparation and maintenance of brain slices

All of the experimental methods used in the conduct of this study followed protocols approved by the Institutional Animal Care and Use Committee of SUNY Downstate Medical Center and the U.S. Surgeon General's Human and Animal Research Panel. All of the experiments used acute coronal slices (450 μm) of rat somatosensory neocortex that were prepared from Sprague-Dawley rats (P21-30) as previously described in detail [Benardo and Prince, 1982]. Slices were placed in an interface recording chamber (Fine Science Tools, Foster City, CA) and maintained at $30 \pm 1°C$. Slices were superfused continuously (~ 1 mL/min) with standard physiological saline, which was composed of (in mM): NaCl 124, KCl 2-5,

$MgCl_2$ 1.6, $CaCl_2$ 2, $NaHCO_3$ 26, D-glucose 10, continuously oxygenated with 95% O_2 and 5% CO_2 (pH 7.35-7.40). All slices were allowed to equilibrate in the experimental chamber for 1-2 h before recording.

Damaged slices were prepared as previously described [Yang and Benardo, 1997]. Initial preparation was identical to that of intact slices. Randomly selected slices were positioned under a dissecting microscope while in ice-chilled saline. Microknives, which consisted of a razor blade fragment held with a hemostat, were used to make a cut parallel to the pial surface that ran the length of the slice at a sub-pial depth of ~450-500 μm. The cut was placed in layer II-III and dendrotomized layer V pyramidal cells. The isolated superficial strip, which included cortical layers I, II, and a portion of III, was discarded. The remaining deep portion (i.e., the "damaged slice"), which comprised lower layer III to layer VI and deep white matter, was then transferred to the experimental recording chamber.

All drugs were delivered through the perfusate. The nootropics aniracetam, 1-BCP, cyclothiazide, IDRA 21 were purchased from Tocris Cookson (Ballwin, MO). Stocks of all nootropic agents were prepared in dimethylsulfoxide (DMSO). Dilutions were made before each experiment and the highest DMSO concentration used was 0.1%. CNQX (6-cyano-7- nitroquinoxaline-2,3-dione) was also obtained from Tocris Cookson. Fluoro-Jade B was purchased from Histo-Chem (Jefferson, AR). Levetiracetam was generously provided by Dr. Helen Valsamis, Department of Neurology, SUNY Downstate Medical Center. All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Electrophysiological recordings

Standard intracellular and extracellular techniques were used to record neuronal activity in layer V of neocortex (Fig 1A and 1E). Membrane potentials were measured using a high impedance amplifier operating in current-clamp mode (AxoClamp 2B, Axon Instruments, Foster City, CA, USA). Microelectrodes were pulled from 1-mm thin-walled, fiber-filled capillaries. For intracellular recordings of individual neurons, electrodes were filled with 2 M potassium acetate and had tip resistances of 60-80 MΩ. For extracellular recordings of field potentials, electrodes were filled with 1 M NaCl and had tip resistances of 2-5 MΩ. Intracellular recordings were made from layer V pyramidal cells, which were identified by their physiologically properties [Yang and Benardo, 1997]. All data signals were digitized at 47 kHz via a 14-bit PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Elmont, NY) and stored on VHS tape for post hoc analysis or recorded directly to computer hard disk using pCLAMP 9.0 software (Axon Instruments, Foster City, CA).

Neural responses were evoked by stimulating slices with cathodal shocks (10-160 μA; 100 μs duration) delivered at low frequency (0.1 Hz) via sharpened, bipolar, tungsten-coated electrodes placed lateral to the recording site at the border of layer VI and deep white matter. Stimulus intensity was systematically varied to determine threshold values for both excitatory postsynaptic potentials (EPSPs) and action potentials (APs). Due to the variability in threshold values between slices, comparisons across treatment groups used "relative" stimulus values that were normalized to the EPSP-threshold value of each slice (i.e., minimal stimulus needed to recruit small, unitary EPSPs).

For LTP experiments, long-term potentiation of excitatory synaptic events was produced by four tetanic trains of 100 Hz stimulation (1 sec duration bursts) applied at 20 sec intervals at the test stimulus value (adjusted to evoke ~50% of the maximal EPSP response), or by using theta-burst stimulation. The theta-burst stimulation protocol consisted of 10 bursts of four pulses at 100-Hz, delivered at 200 ms interburst intervals.

For paired-pulse stimulation (PPS) experiments, pairs of stimuli were applied at the EPSP test stimulus value. The inter-stimulus interval (ISI), which is time between the first and second stimulus pulses, was varied systematically as follows: 20 to 100 ms in 10 ms increments, followed by 150, 200, 250, 300, 400, 600, 800, 1000, and 1200 ms. Paired pulses were applied every 15 sec (i.e., at a low frequency of 0.067 Hz). The peak amplitudes of the first (E1) and second (E2) field potentials of each pair were measured, and the paired-pulse ratios (PPR) were calculated as E2/E1. PPR values >1 were classified as paired-pulse facilitation (PPF), whereas PPR values <1 were classified as paired-pulse depression (PPD).

Histology

At the end of each electrophysiology experiment, slices were recovered from the recording chamber and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brain slices were stained with Fluoro-Jade B (FJB), an anionic fluorecein derivative which selectively labels degenerating neurons with high affinity [Schmued and Hopkins, 2000]. Slices were rinsed in phosphate buffered saline (PBS) two-times for 5 min each. Slices were then incubated in a NaOH bath (1% NaOH in 80% ethanol) for 5 min and then placed in 70% ethanol for 2 min. Sections were then rinsed in distilled water and immersed in 0.06% KMnO4 (120 mg of KMnO4 in 200 mL of dH_2O) for 5 min. Slices were then rinsed in distilled water for 10 min before immersion in FJB staining solution. All subsequent steps were carried out in the dark. A 0.0004% FJB staining solution was prepared from a 0.01% stock (Histo-Chem, Inc.) diluted with a 0.1% acetic acid vehicle (96 µL glacial acetic acid in 96 mL of dH_20). Slices were immersed in the staining solution for 20 min and then rinsed three times in distilled water for 10 min each. The slices were mounted on slides and then cleared with HistoClear (1 min) before coverslipping with DPX (Sigma Chemical Co., St. Louis, Mo), a nonfluorescent, nonaqueous plastic mounting media. Slices were viewed under epifluorsence using confocal microscopy (Zeiss LSM 510 and Axioskop 2) with a blue spectrum light source (450-490 nm) and barrier notch filter for fluorescein (515-565 nm). Slices were examined for the presence of FJB-positive cells, which were identified as such if they displayed a concave cell body and bright fluorescence throughout the cell soma and nucleus [Rocha et al., 2004]. Cell counts of FJB-positive neurons were made from four adjacent fields in each slice.

Data Analysis

All data are presented as means ± SE. Standard errors for treatment group data (i.e. percentages) were calculated as $\sqrt{p(1-p)/n}$, where p is the proportion of samples showing a given effect (e.g., epileptiform activity) and n is the number of samples. Comparisons across treatment groups was performed using the chi-square (χ^2) statistic and Fisher's exact test, except where noted. Significance of changes within individual slices was determined using paired Student's t-test, which each slice serving as its own control. For histological data, statistical significance was determined using one-way ANOVA and Dunnett's post-hoc test for multiple comparisons. For all tests, $p<0.05$ was deemed significant.

Results

Of the agents tested, only the nootropic compound IDRA 21 passed all three tests of efficacy. Our findings indicated that post-injury treatment of neocortical slices with IDRA 21 protected both neural circuit function and cell survival. Application of IDRA 21 to traumatized slices within 30 min *following* injury for 1 h increased seizure-threshold by nearly two-fold, prevented injury-induced cell necrosis, and preserved the ability of cortical circuits to support LTP. The other nootropic agents failed to pass the first test, exhibiting no protective actions against injury-induced epileptogenesis. We found that levetiracetam was superior to IDRA 21 in protecting against injury-induced epileptogenesis by significantly reducing the occurrence of posttraumatic epileptogenesis and also increasing seizure-threshold. However, levetiracetam was significantly less effective in protecting damaged slices against trauma-induced necrotic cell damage. A detailed description of these findings is presented below.

Nootropic agents: Protection against trauma-induced neuropathophysiologies (epileptogenesis)

We first examined the effectiveness of the nootropic drugs to prevent the development of trauma-induced neuropathophysiologies, specifically epileptiform activity. Our previous work showed that 50-60% of traumatized neocortical brain slices exhibit abnormal activity that is characterized by epileptiform bursts of action potential (AP) firing that occur on an all-or-none basis [Yang and Benardo, 1997]. In the neocortex, one hallmark of normal physiological activity is that synaptically evoked responses exhibit graded input-output relationships and trigger single AP discharges. This is illustrated in Figure 1A, which shows a recording from a neocortical layer V pyramidal neuron in an intact slice preparation. A low-intensity stimulus (here, 20 μA) evokes a small, unitary excitatory postsynaptic potential (EPSP), i.e., at EPSP-threshold. As stimulus intensity is increased, the amplitude of EPSPs steadily increases, ultimately reaching sufficient strength to trigger a single AP. Further increases in stimulus intensity still trigger only single (or sometimes double) AP discharges, but not repetitive AP bursts, presumably due to recruitment of recurrent synaptic inhibition that prevents burst firing.

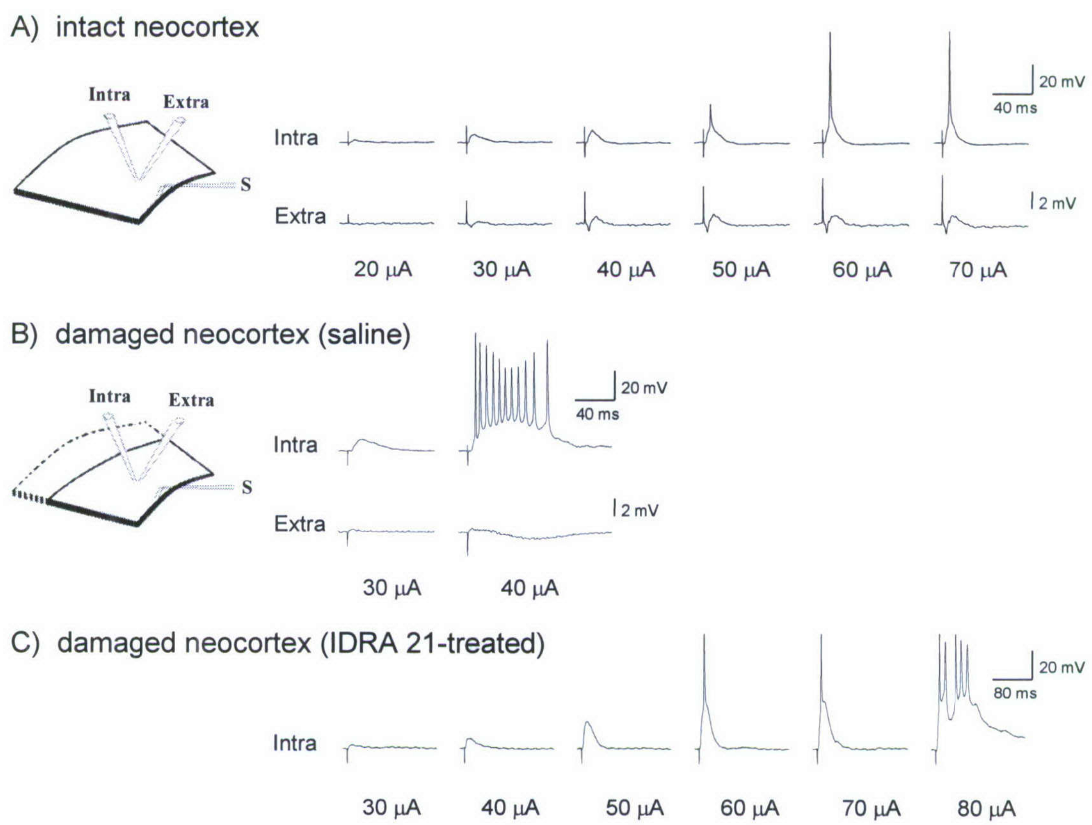

Figure 1 Post-injury treatment of traumatized neocortical slices with IDRA 21 inhibits epileptiform responses. A) Intact, uninjured slices displayed normal response patterns to external stimuli (input-output relationships). Stimulus intensity values are indicated below traces. B) "Damaged" neocortical slices which were experimentally injured by removal of the superficial cortical layers (layers I, II, and part of III) became hyperexcitable, exhibiting abnormal activity characterized by non-graded synaptic responses that triggered epileptiform firing in an "all-or-none" manner. C) Damaged slices that were treated with IDRA 21 post-injury exhibited graded responses, single AP discharges, and significantly higher seizure-thresholds. Intra = intracellular recording; extra = extracellular recording; S = stimulating electrode.

In contrast, damaged cortical slices can become hyperexcitable, exhibiting non-graded synaptic responses that trigger epileptiform AP firing, as shown in Figure 1B. A small elevation in stimulus intensity (+10 μA, in this example) beyond EPSP-threshold triggers epileptic bursts of APs in an all-or-none manner. This activity consists of large, prolonged depolarizations on which three or more AP spikes are superimposed, and is synchronized with simultaneously recorded extracellular population potentials.

This response pattern is characteristic of the paroxysmal depolarization shift (PDS) observed in epileptic foci [Prince and Tseng, 1993; Schwartzkroin, 1995]. We found that 54.7 ± 6.8% (n=53) of damaged slices were hyperexcitable, exhibiting a PDS with bursts of AP spikes that are synchronized with extracellular population potentials The majority of these slices (86%) also showed non-graded input-output responses, with low-intensity stimuli evoking small EPSPs and slightly stronger stimuli evoking all-or-none epileptiform discharges.

All four nootropic agents were examined for their ability to prevent or inhibit injury-induced hyperexcitability and epileptogenesis. Within 30 min after injury, individual slices were exposed to one of the four nootropic agents through the bathing media. Each drug was applied for 1 h and then washed out with control saline. After drug wash-out was complete (~1-2 h), intracellular recordings were used to assess slice excitability. The nootropics were each applied in the following concentrations (i.e., doses): aniracetam, 1.0-2.5 mM; 1-BCP 1.0-2.0 mM, IDRA 21, 200-400 μM; and cyclothiazide 100 μM. Electrophysiological recordings revealed that nootropic drug-treated damaged slices also developed epileptiform activity, in proportions similar to non-treated slices. The percentages of nootropic-treated slices that exhibited abnormal firing were as follows: IDRA 21, 50.3 ± 13.3%, (n=24); aniracetam, 43.8 ± 8.7% (n=32); 1-BCP 63.6 ± 14.5% (n=11); and cyclothiazide 75.0 ± 9.3% (n=4). None were significantly different from non-treated damaged slices (Fig 2A). In these slices, discharges with PDS-like depolarizations and repetitive bursts of APs could be evoked with stimuli of sufficient intensity. These findings indicate that none of the nootropics tested were able to reduce the occurrence of injury-induced epileptogenesis.

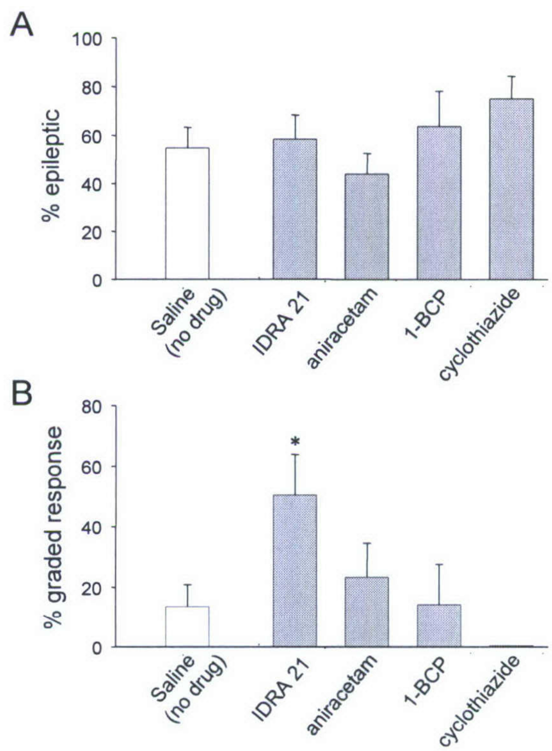

Figure 2 Frequency of the development of epileptiform and graded responses in slices treated with nootropic agents. A) Post-injury treatment with nootropics agents did not prevent or reduce occurrence of epileptiform activity in traumatized neocortical slice preparations. B) However, treatment of damaged slices with IDRA 21 did preserve graded synaptic responses in >50% of slices. This effect was attributed to an increase in seizure-threshold in these slices. * P<0.05.

However, closer examination of the data revealed that IDRA 21 increased seizure-threshold in damaged slices (i.e., the stimulus intensity needed to evoke epileptiform firing). This appeared to have the additional effect of preserving graded synaptic responses. As shown in Figure 1C, stimuli applied to IDRA 21-treated damaged slices at intensities below seizure-threshold levels evoked EPSPs in a graded manner. As stimulus intensity was increased, EPSPs increased steadily in amplitude until single APs were triggered, similar to normal responses in intact slices. However, further elevations in stimulus intensity ultimately exceeded seizure-threshold and evoked epileptiform responses. To assess this effect in greater detail, we calculated the proportion of epileptic slices in each drug treatment group that exhibited graded synaptic responses. The results were as follows: non-treated damaged slices, 13.6 ± 7.3% (n=22); IDRA 21, 50.5 ± 13.3% (n=14); aniracetam, 23.3 ± 11.3% (n=14); 1-BCP, 14.3 ± 13.3% (n=7); cyclothiazide, 0% (n=4). Statistical analysis indicated that relative to non-treated slices, only IDRA 21 significantly increased the percentage of damaged slices which exhibited graded synaptic responses (Fig. 2B). As stated above, this appeared to stem from an increase in seizure-threshold induced by IDRA 21 treatment.

To assess this further, we compared seizure-threshold values in non-treated vs. IDRA 21-treated damaged slices (Fig. 3). The seizure-threshold value for each slice was taken as the "relative" stimulus intensity required to evoke epileptiform firing (i.e., normalized to the EPSP-threshold stimulus for the same slice). In non-treated damaged slices, the normalized seizure-threshold value was 1.37 ± 0.04 (n=13, range 1.2-1.6). This number indicated that in damaged slices, seizure-threshold was, on average, 37% above EPSP-threshold. In IDRA 21-treated slices, seizure-threshold was significantly higher, with a mean value of 2.62 ± 0.24 (n=10, range 1.6-4.0; $p<0.05$, unpaired t-test). The other three nootropics (aniracetam, 1-BCP, cyclothiazide) had no significant effect on seizure-threshold. These results show that IDRA 21 raised the seizure-threshold level in damaged neocortices and, as a result, extended the range of normal physiological responses in the remaining cortical circuits.

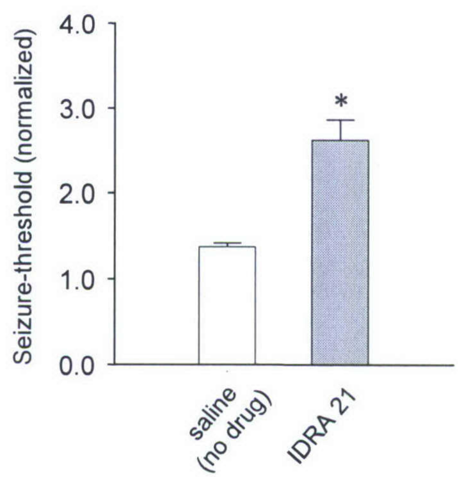

Figure 3 Treatment of damaged slices with IDRA 21 significantly increased seizure-threshold relative to non-drug-treated slices. Seizure-threshold values were taken as the minimum strength stimulus which evoked epileptiform bursts of APs in an individual slice. These values were normalized to the EPSP-threshold value for each slice. *$P<0.05$.

In contrast to IDRA 21, the other nootropic agents failed this first critical test of efficacy, having no effect on epileptogenesis or seizure-threshold. Consequently, based on these results, aniracetam, 1-BCP, and cyclothiazide were withdrawn from further consideration at this time.

Nootropic agents: Effects on synaptic strength

To gain further insight on IDRA 21's mechanism of protection, we examined its effects on intrinsic neuron membrane properties and synaptic excitability. Based on previous findings [Ling and Benardo, 2005], we hypothesized that IDRA 21 may increase GABA-mediated synaptic inhibition by enhancing AMPAR-mediated, synaptic activation of GABAergic inhibitory interneurons. Effects on intrinsic neuron membrane properties were also examined.

The effects of IDRA 21 on intrinsic cell membrane properties were evaluated from intracellular recordings that were made during slice exposure to control saline and then to IDRA 21 (400 μM) (n = 5 cells, from five slices) . The data indicated IDRA 21 did not alter cell membrane properties. There was no significant difference in cell resting potential, input resistance or membrane time constant values in the absence or presence of IDRA 21 ($p>0.05$):

	Resting potential	Input Resistance	Time Constant
Control saline	-70.6 ± 1.3 mV	22.5 ± 0.8 MΩ	8.7 ± 0.2 ms
IDRA 21 (400 μM)	-72.3 ± 1.1 mV	21.9 ± 1.2 MΩ	9.1 ± 0.3 ms

IDRA 21 also had no effect on the intrinsic excitability of neurons, as it did not alter neuronal responses to either depolarizing or hyperpolarizing current pulse injections (Fig. 4A). In addition, IDRA 21 did not change neuronal responses to evoked synaptic inputs, as externally applied high-intensity stimuli (i.e., > AP-threshold) evoked single APs in both the absence and presence of IDAR 21 (Fig. 4B).

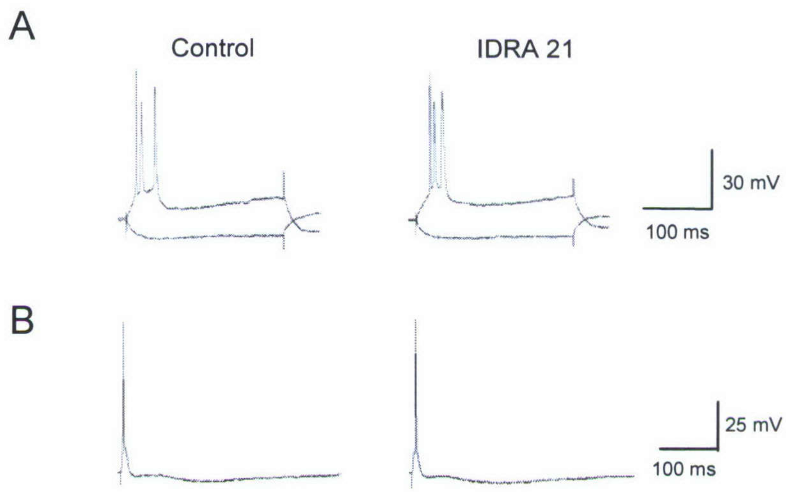

Figure 4 IDRA 21 does not alter the intrinsic cell membrane properties or evoked responses of neocortical pyramidal neurons. A) Neuronal responses to hyperpolarizing (-0.5 nA) and depolarizing (0.4 nA) current pulse injections that were recorded in control saline remained unchanged following subsequent exposure of slices to IDRA 21 (400 μM). B) In control saline, electrical stimulation of slices evoked EPSPs which triggered an action potential discharge followed by fast and slow IPSPs (inhibitory postsynaptic potentials). Exposure of slices to IDRA21 did not alter evoked synaptic responses.

Next, we conducted a detailed examination of the effects of IDRA 21 on evoked synaptic events. In both intact and damaged slices, evoked field EPSPs (fEPSPs) exhibited early (Fig. 5A, single arrow) and late components (Fig.5A, double arrows). Bath application of CNQX (10 μM), a non-NMDA receptor antagonist, blocked the early component (n=3) but not the late component (Fig. 5A, right panel), indicating the former is mediated by AMPA receptors, whereas the latter is mediated by both AMPA and

NMDA receptors. In damaged slices (Fig. 5B), IDRA 21 enhanced the early (AMPA-dependent) component, consistent with its primary action as a positive AMPA receptor modulator. During exposure to IDRA 21, damaged slices also exhibited a late hyperpolarizing potential (Fig. 5B, ★) that was conspicuously absent in control saline, and which may represent a GABA-mediated inhibitory postsynaptic potential (IPSP).

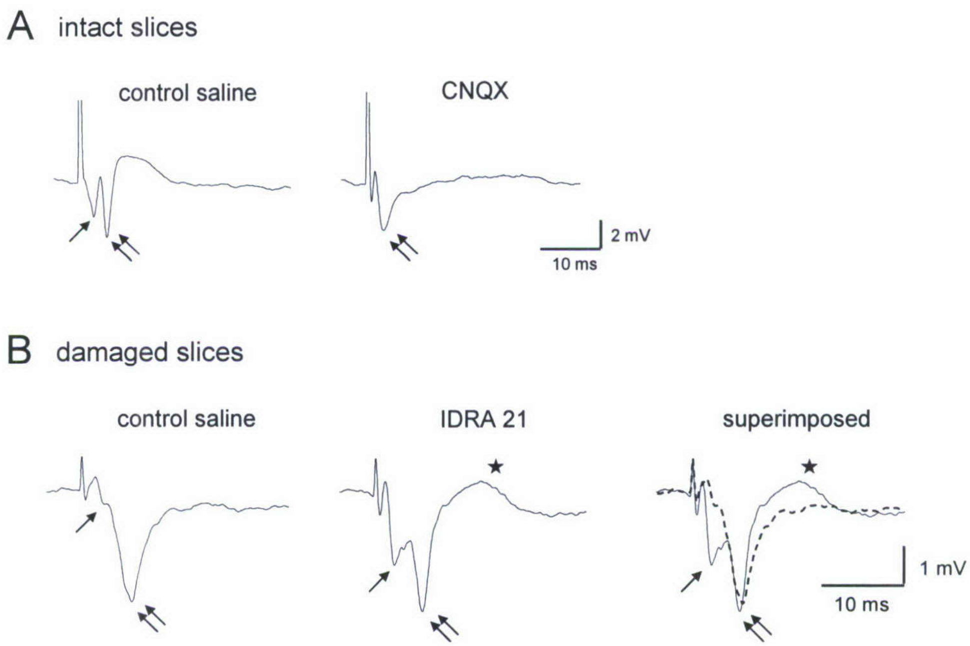

Figure 5 Effects of IDRA 21 on evoked synaptic events. A) Field EPSPs (fEPSPs) evoked in intact slices exhibited early (single arrow) and late components (double arrows). CNQX (10 μM) blocked the early component, but not the late component. B) In damaged slices, IDRA 21 enhanced the early, AMPA-mediated component of fEPSPs as expected. IDRA 21-treated slices also exhibited a late hyperpolarizing potential (★) that may reflect an enhancement of GABA-mediated inhibition.

To further assess the effects of IDRA 21 on synaptic transmission, fEPSPs were evoked using the paired-pulse stimulation (PPS) paradigm, which is a method of assessing changes in synaptic strength [Castro-Alamancos and Connors, 1997; Rozas et al., 2001]. When PPS is applied at inter-stimulus intervals (ISIs) that are within the time frame of inhibitory synaptic events (10 to 100 ms), a depression of the fEPSP late component occurs known as paired-pulse depression or PPD (Figure 6A, left panel; double-arrows). Past studies have shown that PPD involves the recruitment of GABAergic synaptic inhibition, as evidenced by the attenuation of PPD by GABA receptor antagonists [Rozas et al., 2001].

PPS was examined in both intact and damaged slices and showed similar response patterns over ISI's from 20 to 1200 ms (Fig. 6B, left graph). At short ISI times, PPS evoked strong PPD in which the second event was significantly smaller in amplitude than the first. As the ISI time was lengthened, PPD diminished and ultimately switched to paired-pulse facilitation (PPF) in which the second event was now significantly larger than the first. The magnitude of PPF reached a peak value at an ISI of ~40 ms. As ISI time was further increased, responses switched back to PPD. A comparison of PPS responses between intact (n=12) and damaged slices (n=16) revealed that PPD was significantly lower (i.e., less depression) in damaged slices at short ISIs of 20 to 30 ms (Fig. 6A-B). This suggests damaged slices have a loss of synaptic inhibition (i.e., are "disinhibited"), which is in agreement with our past findings [Yang and Benardo, 1997].

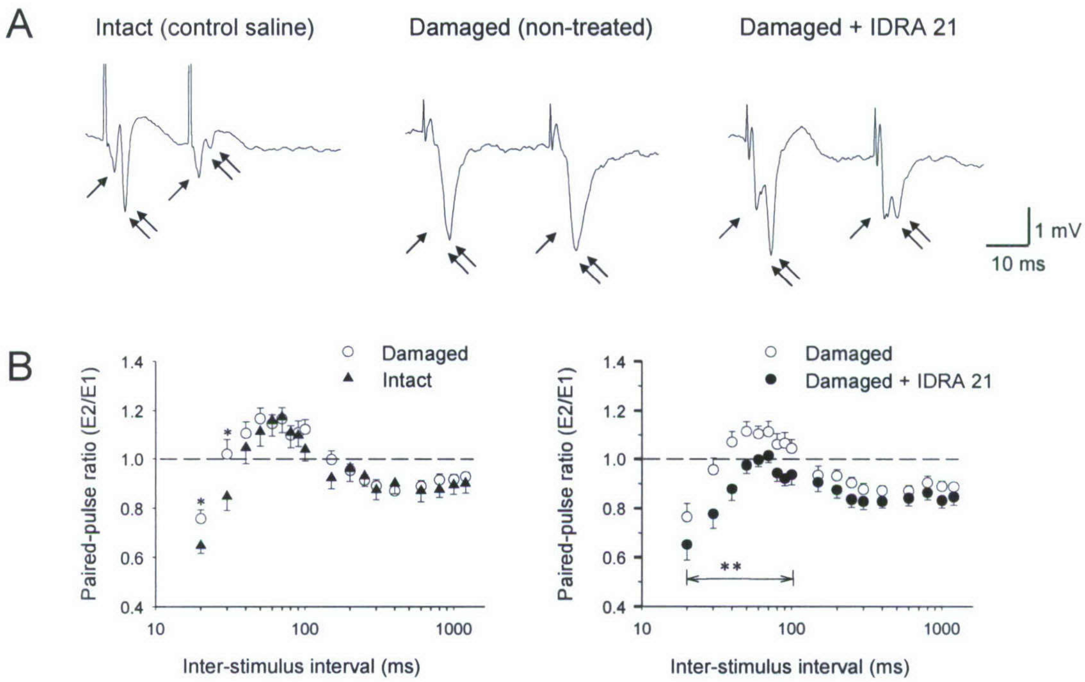

Figure 6 Effect of IDRA 21 on paired-pulse stimulation of evoked fEPSPs. A) Paired-pulse stimulation (PPS) of intact slice preparations (left panel) produced depression of the late component of fEPSPs, i.e., paired-pulse depression (PPD), at short ISI times. PPS of damaged slices (middle panel) at short ISI's caused significantly lower levels of PPD. IDRA 21 treatment led to a significant increase in PPD in damaged slices, suggesting it may have induced an augmentation of GABAergic inhibition. (*, ** $P<0.05$).

We then examined PPS in damaged slices treated with IDRA 21 (200 μM). At ISIs from 20 to 100 ms, PPD was significantly greater in IDRA 21-treated damaged slices (n=11; unpaired t-test) than in non-treated slices (Fig. 6A-B). In addition, PPF was effectively suppressed in IDRA 21-treated damaged slices (Fig. 6B, right graph). Together, these findings suggest IDRA 21 induces an enhancement of GABA-mediated synaptic inhibition in damaged slices. This may represent one mechanism of its neuroprotective actions. However, it is unclear whether this enhancement stems from potentiation of inhibitory synapses, protection of GABAergic inhibitory interneurons, or a combination of both, or whether IDRA 21 engages other protective pathways as well.

Nootropic agents: Protection against trauma-induced cell necrosis

The next set of tests assessed IDRA 21's ability to protect against injury-induced cell necrosis. Histological analysis was conducted with Fluoro-Jade B (FJB), a fluorescent marker that specifically stains degenerating neurons. Damaged slices exhibited substantial numbers of FJB-positive neurons (164.9 ± 26.8 cells per slice, n=10) within 1-3 h after injury, suggesting a rapid progression of cell necrosis (Fig. 7, top, middle panel; arrows). The mean number of FJB-positive cells in IDRA 21-treated damaged slices was 4.3 ± 1.2 cells per slice (n = 11), a significant reduction in comparison to non-treated injured slices. A comparison with intact slices showed that the number of necrotic cells in IDRA 21-treated damaged slices closely matched (and were actually lower than) those in intact, uninjured neocortical slices (13.3 ± 3.9 cells per slice, n=8). This suggests IDRA 21 effectively prevents the spread of trauma-induced neuronal damage, at least in this brain slice preparation. One interesting finding was that intact slices treated with IDRA 21 also exhibited very low levels of necrotic neurons (3.0 ± 1.2 cells, n=5), confirming that IDRA 21 is not neurotoxic and indeed may benefit neurons in healthy brain tissue. Taken together, these results suggest IDRA 21 may provide an effective means of isolating injured brain regions by preventing, or at least containing, the spread of pathological damage.

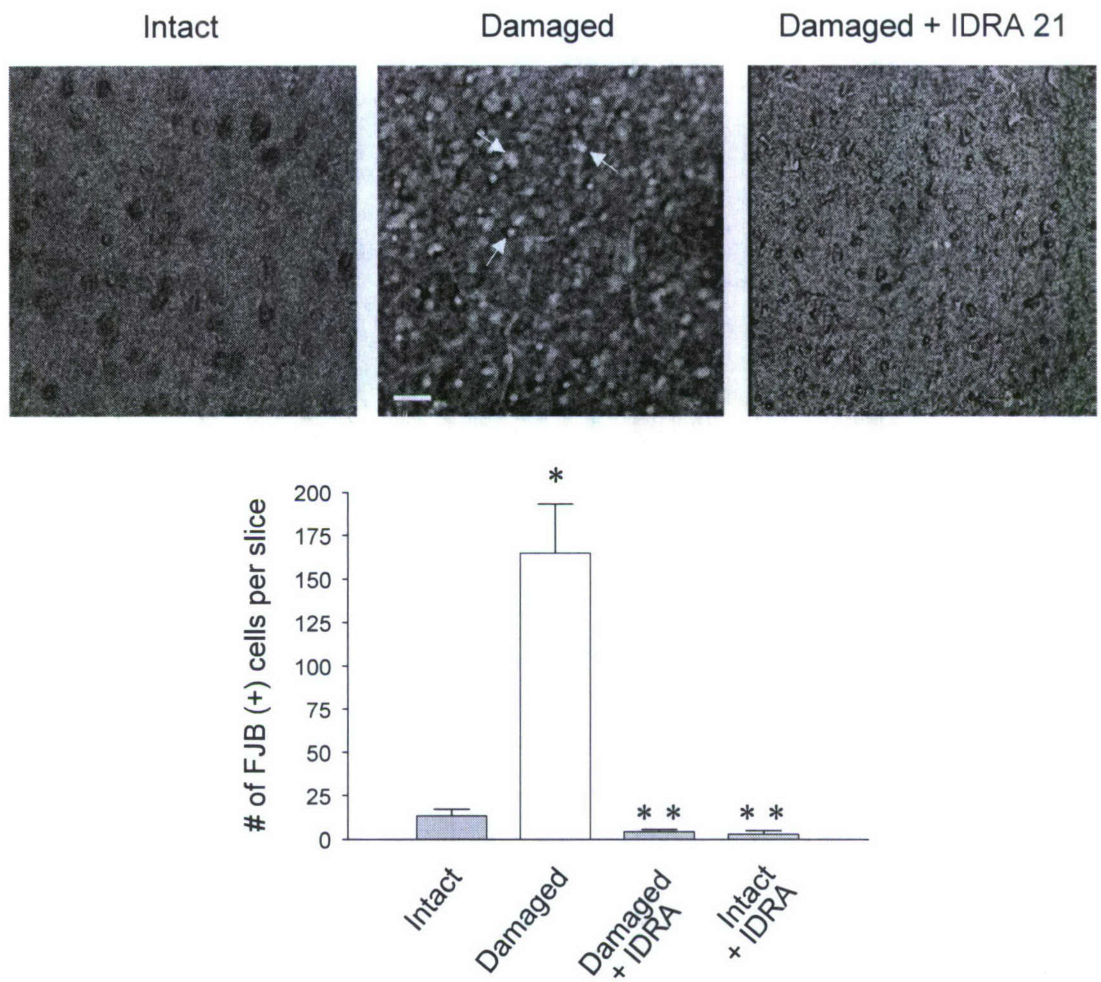

Figure 7 IDRA 21 prevents injury-induced cell necrosis in rat neocortical slices. Damaged slices that were subjected to superficial cortical injury exhibited significant levels of Fluoro-Jade B positively stained cells. Post-injury treatment of damaged slices with IDRA 21 reduced the number of FJB-positive cells per slice, matching levels observed in intact slice preparations. Arrows indicate typical cells identified as FJB-positive. Scale bar = 50 μM. *P<0.05, relative to intact slice preparations. **P<0.05, relative to damaged slice preparations.

Nootropic agents: Preservation of normal neurophysiological processes

Following traumatic injury, cortical circuits routinely lose the ability to support activity-dependent long-term potentiation (LTP) of synaptic excitatory transmission [Albensi and Janigro, 2003; Sanders et al., 2000], the form of synaptic plasticity believed to be the substrate of memory and learning. This was confirmed in our in vitro model of traumatic brain injury. In 18 intact slice preparations, tetanic stimulation induced a persistent potentiation of field excitatory postsynaptic potentials (fEPSPs), resulting in a $59.5 \pm 11.5\%$ ($p<0.05$, paired t-test) increase in fEPSP magnitude relative to baseline values. In contrast, damaged slices were unable to support LTP, as tetanic stimulation failed to alter fEPSPs evoked in any of the damaged slice preparations tested (Fig. 8, left panel). In these slices, fEPSPs recorded post-tetanus were statistically similar to baseline events recorded in the same slices (mean change: $-12.5 \pm 11.5\%$ below baseline; $n=11$, $p>0.05$).

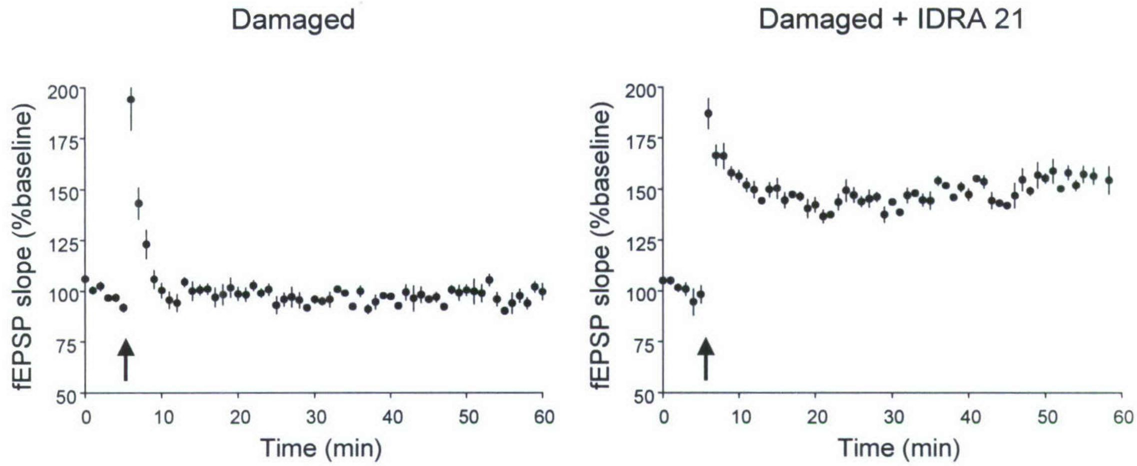

Figure 8 IDRA 21 treatment protects the ability of injured neocortical slices to support LTP. (Left panel) Tetanic stimulation of damaged neocortical slices failed to induce a persistent, long-term potentiation of fEPSPs recorded in layer V. (Right panel) In contrast, damaged slices that were treated post-injury with IDRA 21 and then subsequently tetanized with high-frequency stimulation did express long-term potentiation of excitatory synaptic potentials. (Arrow indicates time point of tetanus application).

We then examined the effects of IDRA 21 treatment on LTP. The results showed that IDRA 21-treated damaged slices retained the ability to support activity-induced LTP. In IDRA 21-treated slices (n=7), tetanic stimulation produced a persistent potentiation of field EPSPs that led to a 52.5 ± 5.9% increase in fEPSP magnitude (Fig. 8, right panel). Thus IDRA 21 treatment preserved the neural circuit and cellular mechanisms that underlie LTP, suggesting it may help protect the critical physiological processes that support normal cognitive operations.

The combined findings of these studies prompted us to propose a series of supplemental experiments to investigate the neuroprotective effects of levetiracetam, given its role as a proven anticonvulsant drug and its structural relationship to certain nootropic agents. These were performed as an extension of our original research proposal and the results are summarized below.

Supplemental Studies

Levetiracetam: Protection against trauma-induced neuropathophysiologies (epileptogenesis)

Past studies performed in our laboratory suggest that anticonvulsant drugs may also be neuroprotective, especially in inhibiting posttraumatic epiletigenosis [Yang and Benardo, 2000]. In addition, recent reports have shown that levetiracetam improves functional and histological outcomes in experimental models of closed-head injury [Wang et al., 2006]. As such, we were understandably curious whether levetiracetam, an FDA-approved antiepileptic drug, was also neuroprotective in our model of superficial cortical injury. We hypothesized that levetiracetam might exert even stronger protective effects than the nootropics, due to the combination of its established anticonvulsant actions and its structural similarity to the pyrrolidione nootropics.

Using the same treatment protocol, we examined the effects of post-injury applied levetiracetam (250-500μM) on traumatized cortical slices. The data suggest that post-injury treatment of brain slices with levetiracetam was superior to IDRA 21 in preventing the development of trauma-induced epileptogeneis. Only 27.8 ± 10.6% (n=18) of levetiracetam-treated slices exhibited epileptiform activity, a 50% reduction from the levels observed in non-treated slices, 54.7 ± 6.8%, n=53 (Fig. 9). In addition, levetiracetam-treatment also raised seizure-threshold in damaged slices (Fig. 9, right-hand panel). The relative stimulus intensity required to evoke epileptiform bursts in levetiracetam-treated slices was 2.66 ±

0.24 (n=6; range: 1.30 - 3.30), which was significantly higher than the value reported earlier for non-treated slices, 1.37 ± 0.04 (n=13; unpaired t-test). As a result of these findings, we are continuing our investigations of levetiracetam in hopes of elucidating the precise mechanisms of its protective effects on cortical neural circuit physiology.

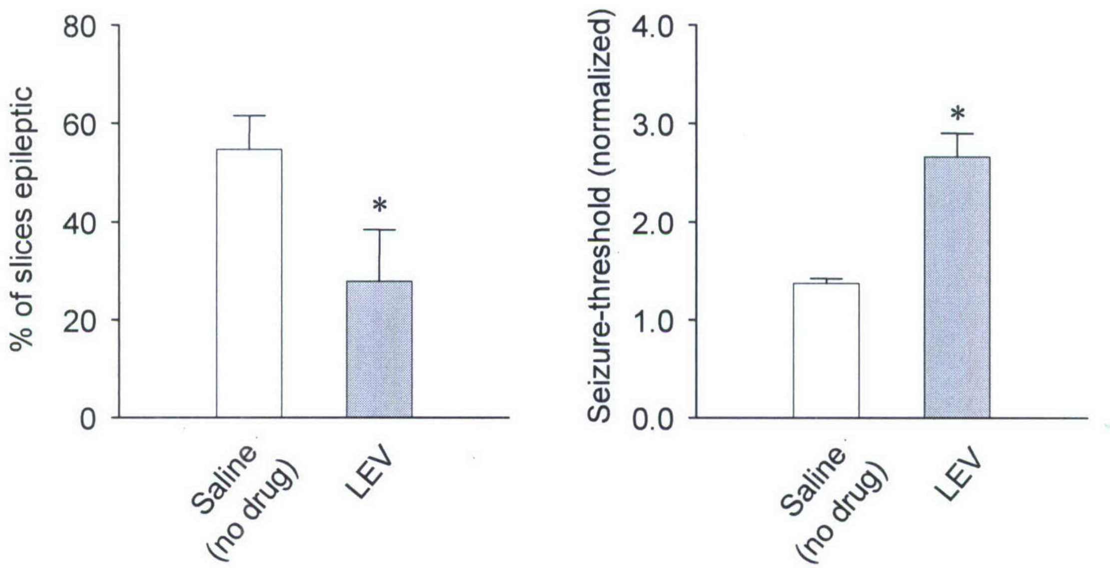

Figure 9 Post-injury treatment of traumatized neocortical slices with levetiracetam reduced the percentage of damaged slices that become hyperexcitable and exhibit epileptiform activity (left panel). Levetiracetam (LEV) treatment also induced a significant increase in seizure-threshold relative to non-treated injured slices (right panel). *$P<0.05$.

Levetiracetam: Protection against trauma-induced cell necrosis

We assessed the effects of levetiractam treatment on trauma-induced cell necrosis. The data indicated that levetiracetam (250-500 μM) reduced the levels of neuron loss in damaged slice preparations, though it was substantially less effective than IDRA 21. In levetiracetam-treated slices, the number of FJB-positively stained cells was 91.4 ± 13.7 cells per slice (n=11), which represents a significant reduction (- 42%) in cell necrosis relative to non-treated damaged slices. However, when compared to intact slice preparations, these levels were significantly higher than those in uninjured neocortex (reported above), suggesting levetiracetam reduces, but does not prevent, trauma-induced cell necrosis (i.e., unlike IDRA 21). Nonetheless, the results indicate that levetiracetam significantly promotes neuron survival when administered after cortical injury.

Levetiracetam: Preservation of normal cortical function (long-term potentiation)

We assessed levetiracetam's ability to preserve the capacity of damaged slices to support LTP. As shown in Figure 10, application of tetanic stimulation failed to potentiate excitatory synaptic transmission in any of the levetiracetam-treated damaged slices examined (n=5). Comparisons of fEPSPs recorded before and after application of tetanus showed there was no significant change in the magnitude of fEPSPs (mean change: +3.7 ± 0.1% relative to baseline; $p>0.05$). These findings indicate that levetiracetam did not protect or promote the ability of neural circuits in damaged neocortical slices to support long-term potentiation. Thus, unlike IDRA 21, levetiracetam may not be effective in preserving the physiological processes that support cognitive function.

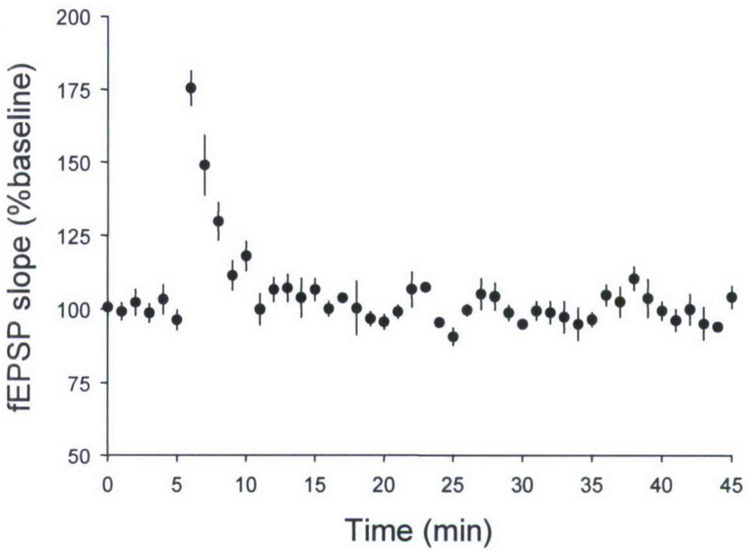

Figure 10 Effect of levetiracetam treatment on the ability of injured slices to support LTP. Tetanic stimulation of levetiracetam-treated damaged slices failed to elicit long-term potentiation of evoked fEPSPs.

Summary and Discussion

The results of this study showed that the nootropic agent IDRA 21 was the only compound tested that fulfilled all three criteria of efficacy. Although the underlying mechanisms of its protective actions are still unclear, our findings suggest that one contributing factor may be an IDRA 21-induced enhancement of synaptic GABAergic inhibition, possibly mediated through actions on inhibitory interneurons [Ling and Benardo, 2005]. This mechanism would serve to preserve the normal balance between synaptic excitation and inhibition in traumatically injured cortices and, in turn, help maintain normal neural circuit function. This action would help counter any injury-induced changes in synaptic transmission [Yang and Benardo, 1997] and prevent or suppress posttraumatic epileptogenesis. This would also help preserve other normal physiological processes, such as LTP, which IDRA 21 has also been shown to promote in hippocampal circuits [Arai and Lynch, 1996]. Its protective qualities against cell necrosis may also derive, in part, from these actions, which would limit the spread of hyperexcitation to otherwise healthy, uninjured brain regions. This would help reduce or prevent excitotoxic cell damage. Other positive actions of IDRA 21 may also contribute to its neuroprotective effects. Past studies suggest that positive modulators of AMPAR function, such as the nootropics, potentiate the release of neurotrophins, such as BDNF, NGF, and NT3 [Lauterborn et al., 2000; Lauterborn et al., 2003; Lockhart et al., 2007]. This may provide protection against other types of brain injury, as suggested by data from animal studies that show a protective action by positive AMPAR modulators against ischemia-induced hippocampal cell death [Roger et al., 2004].

However, this does not explain the inability of the other three nootropic agents to provide similar protection. The reasons for their ineffectiveness are unclear at the present time. Our past studies showed that both aniracetam and cyclothiazide enhance GABAergic inhibitory synaptic transmission in neocortical circuits [Ling and Benardo, 2005], so our expectation was that they would have some protective actions. However, other studies have shown that different AMPAR modulator agents, even those of the same chemical family, have highly variable effects and efficacy, owing to different physical properties, receptor affinities, and sites of actions [Black, 2005; Impagnatiello et al., 1997; O'Neill et al., 2004]. For example, aniracetam is considered a safe agent with low toxic potential, but has only modest therapeutic effects clinically. As such, aniracetam may lack sufficient potency to be a truly effective neuroprotectant. Cyclothiazide is recognized as a very potent AMPAR modulator, but this could have drawbacks, as recent studies suggest it may be epileptogenic and neurotoxic to hippocampal circuits [Qil et al., 2006]. Although our examinations of cyclothiazide have not revealed deleterious effects to neocortex, potential toxicity to other brain regions must be taken into consideration. In contrast, past studies suggest that IDRA 21 has very low toxic potential, which may be due, in part, to its actions as a partial AMPAR modulator [Impagnatiello et al., 1997].

Levetiracetam's superior efficacy in inhibiting injury-induced epileptogenesis may stem from direct actions on synapses that counter pathological changes in synaptic function [Lynch et al., 2004]. Recent studies suggest levetiracetam may also suppress some glutamateric synapses by acting as a negative modulator of AMPARs [Carunchio et al., 2007]. These actions could work synergistically to counter the sources of injury-induced disinhibition that may lead to posttraumic epileptogenesis, such as the loss of synaptic inhibition and the Ca^{2+}-mediated enhancement of synaptic excitation [Yang and Benardo, 1997; Yang and Ling, 2007;]. However, levetiracetams's reportedly modest effects on AMPAR function (which distinguishes it from other pyrrolidiones) may account for its relatively weaker protective actions against injury-induced cell necrosis. Additional studies will be needed to fully address these issues. Nonetheless, levetiracetam could serve as a useful adjunct to other therapeutic agents, and thus aid the development an effective, pharmaceutical-based brain tourniquet.

Conclusion

The findings of this study support the feasibility of a "brain tourniquet" approach to TBI, showing that it may be possible to physiologically isolate damaged brain regions and thus preserve both normal neural function and cell survival. Our data suggest that such a system may be achievable though rational drug-based therapies that could be administered *after* injury in the acute, post-injury phase of TBI (either singly or possibly in combination). One promising agent that was identified in this study is the nootropic drug IDRA 21. Another agent with potential is levetiracetam, an FDA-approved antiepileptic drug that might serve as a useful adjunct to help further enhance neuroprotection.

However, while the data from this exploratory study are encouraging, the in vitro brain slice preparation employed still represents a reductionist system and, as such, does not model the full spectrum of responses to TBI by the intact brain, or the global effects of TBI on neurological and behavioral function in humans. As such, further studies will be needed to assess the efficacy of this approach in vivo by using whole-animal models that more closely parallel brain injury sustained by warfighters. A Phase II study incorporating whole-animal TBI models will provide further rigor in pre-clinical testing via combined physiological, histological, and behavioral assessments that are a prerequisite to clinical trials of a deployable brain tourniquet system.

Publications

Yang, L., Afroz, S., and Ling, D.S.F. Neuroprotection by the nootropic agent IDRA 21 in an acute rat brain slice model of traumatic cortical injury. Society for Neuroscience Abstracts, 2008.

Yang, L., Afroz, S., and Ling, D.S.F. The nootropic agent IDRA 21 protects against acute cortical injury in a rat neocortical slices (manuscript in preparation).

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